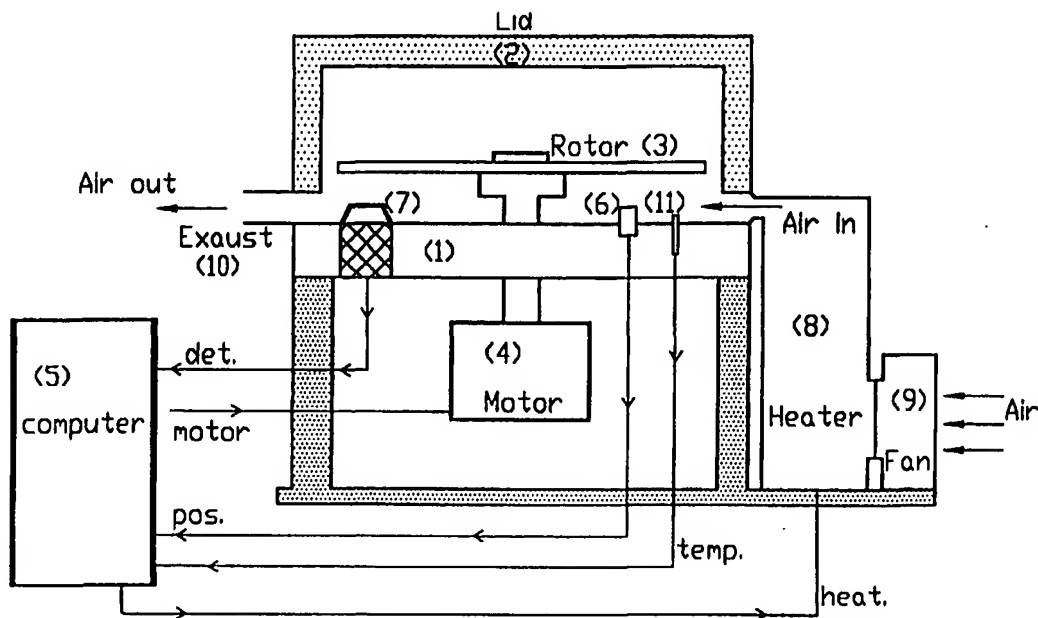


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| (21) International Application Number: PCT/AU98/00277 (22) International Filing Date: 21 April 1998 (21.04.98) (30) Priority Data: PO 6529 30 April 1997 (30.04.97) AU (71)(72) Applicants and Inventors: CORBETT, John, Michael [AU/AU]; 39 Renwick Street, Drummoyne, Sydney, NSW 2047 (AU). CORBETT, John, Michael, Jr. [AU/AU]; 5/24 Yarranabee Road, Darling Point, Sydney, NSW 2027 (AU). | | (81) Designated States: AU, CA, JP, NZ, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> |

(54) Title: TEMPERATURE CYCLING DEVICE AND METHOD**(57) Abstract**

The present invention provides an apparatus and method for the amplification and real time detection of specific DNA fragments, using Polymerase Chain Reaction, Ligase Chain Reaction or any other amplification or hybridization technology. The method of the present invention involves centrifugal loading of DNA samples and reagents into a rotor which is sealed to eliminate contamination. This sealed rotor is then heated and or cooled, a detection means monitors the fluorescence of each sample during temperature transitions. The method of the present invention allows rapid processing of a large number of samples.

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TEMPERATURE CYCLING DEVICE AND METHOD DESCRIPTION

BACKGROUND OF INVENTION

The present invention relates to a device and method for the rapid cycling
5 of a reaction mix between various temperatures. The device and method are particularly suitable for conducting DNA polymerase or ligase chain reactions, however, can be used for a range of enzymatic reactions, which involve changes of temperature.

In a number of applications such as gene analysis and DNA profiling, it is
10 desirable to multiply the amount of particular nucleic acid sequences present in a sample. For example, a duplex DNA segment of up to approximate six thousand Base pairs in length may be amplified many million fold by means of the polymerase chain reaction (PCR), starting from as little as a single copy.

In this technique a denature duplex DNA sample is incubated with a molar
15 excess of two oligonucleotide primers, one being complimentary to a short strand of the DNA duplex and the other being identical to a second short sequence upstream of it (i.e. more 5'), such that each primer anneals to its complimentary sequence and primes the template-dependent synthesis by DNA polymerase of a complimentary strand which extends beyond the site of annealing
20 of the other primer, through the incorporation of deoxynucleotide triphosphates provided. Multiple cycles of denaturation, annealing and synthesis each afford an approximate doubling of the amount of target sequence, where the target sequence is defined as the DNA sequence subtended by and including the primers. Each cycle is controlled by varying the temperature to permit successive denaturation
25 of complimentary strands of duplex DNA, annealing of the primers to their complimentary sequences and a prime synthesis of new complimentary sequences and a prime synthesis of new complimentary DNA strands. The use of a thermostable DNA polymerase obviates the necessity of adding new enzyme for each cycle, thus allowing automation of the DNA amplification process by
30 thermal cycling. Twenty amplification cycles increases the amount of target sequence by approximately one million-fold (being theoretically 220).

More detailed information regarding the polymerase chain reaction can be found "PCR Protocols – A Guide to Methods and Application" Eds. M.A. Innis, D. H. Gelfard, J.J. Sainskey, T.J. White, Academic Press, Inc. San Diego 1990, the disclosure of which is incorporated herein by reference.

5 In the prior art it has been found that the technique of DNA polymerization requires rapid controlled heating and cooling cycles. The art is replete with incubators and other devices to achieve this end, e.g. PCT/AU90/00560.

10 Typically a device used for PCR consists of a heat conductive material provided with channels adapted to receive vessels in which the reaction is to take place, typically Eppendorf tubes. The heat conductive material is then provided with heating/cooling means.

15 Wittwer *et al* Biotechniques 10 76-82 (1991) state that in commercial units for automated DNA amplification, temperature transition rates are usually no less than 3°C per second when metal blocks or water are used for thermal equilibration and samples are contained in plastic micro-centrifuged tubes. A significant fraction of cycle time is spent heating and cooling the sample, as opposed to being spent at optimal denaturation, annealing and elongation temperature. Extended amplification times of two to four hours are common and
20 long transition times make it difficult to determine optimal temperatures and times for each stage. Instantaneous temperature changes are not possible because of sample, container and cyler heat capacities.

The present inventors have developed a novel device and method, which allows the rapid cycling of a reaction mixture between various temperatures.

25 Accordingly, in a first aspect the present invention consists in a device comprising a chamber containing a rotor provided with a plurality of reaction zones and a plurality of sample loading zones, drive means to drive the rotor thereby applying a centrifugal force to the sample tubes, heating means to raise the temperature within the chamber, and openings through which a coolant gas
30 can be admitted into the chamber.

In a preferred embodiment of the present invention the heating means are positioned within the chamber and will typically be infrared lights, convection heating elements or microwave sources. It is presently preferred, however, that the heating means are convection heating elements. The heating means need not
5 be positioned within the chamber, for example, the heating means may comprise a hot air blower from which a stream of heated air is directed into the chamber.

The coolant gas can be any of a large number of gasses, however, for the sake of convenience and cost effectiveness in most cases the coolant gas will simply be air at ambient temperature. It is, however, possible that in some
10 situations refrigerated air may be used. This may be particularly beneficial where, after the required number of cycles have been conducted, it is desirable to cool the samples to sub-ambient temperatures.

In a preferred embodiment means to open and close the openings are provided.

15 In a further preferred form of the present invention the device will include means for automatically actuating the heating means when the openings are closed and, after a predetermined time, turning off the heating means and opening the openings.

The openings are preferably provided in the top of the chamber and in the
20 side walls of the chamber. Each opening provided in the side wall of the chamber is positioned directly opposite another opening in the side wall of the chamber so that while the rotor is spinning at a constant speed there is no movement of air from the chamber.

In another preferred embodiment of the present invention the reaction
25 zones are glass capillary tubes and the sample loading zones are slots. Typically two sample-loading slots will be provided for each reaction zone thereby enabling the components of the reaction mixture to be kept separate prior to the thermocycling. The loading slots will be in fluid communication with the reaction zone such that upon actuation of the heating means will rapidly heat the
30 contents of the sample within the reaction zone to the required temperature. An increase in the speed of the rotor will result in air being drawn into the chamber

through the openings displacing the heated air previously with the chamber. This will result in a rapid cooling of the contents of the reaction zone. Accordingly, it can be seen that the device of the present invention will enable the rapid cycling between the temperatures routinely used in carrying out the polymerase chain reaction and other enzymatic reactions. The use of the device of the present invention will, therefore, provide an effective method of cycling a reaction mixture between various temperatures.

In yet a further preferred embodiment of the present invention monitoring means to assess the progress of the reaction occurring in the sample tube within the chamber. Typically, this monitoring means will be a fluorescence detector, spectrophotometer, or photometer. This would be particularly useful in monitoring the progress of a number of enzymatic reactions where a change in optical density or fluorescence of the product is observed. Such monitoring means would also be very useful in monitoring PCR reactions. In this case an intercalating dye, such as Ethidium bromide or SYBR® Green, would be added to the reaction mix. When the dye binds to double stranded DNA there is fluorescence. Accordingly, by monitoring the degree of fluorescence in the reaction mixture an assessment as to the number of doublings, which have occurred, can be made. Alternatively, fluorescently labeled probes that hybridize to the DNA could be used.

The cycling between various predetermined temperatures can be automated. This would involve actuation of the heating means, turning off the heating means and increasing the speed of the rotor, decreasing the speed of the rotor and reactivating the heating means. Further, in the situation where monitoring means are provided in the chamber when the reaction has reached a suitable point refrigerated gas may be pumped into the chamber thereby cooling the reaction mixture to a sub-ambient temperature.

Whilst as stated above it is presently preferred to simply provide the coolant has to the chamber by varying the speed of the rotor this coolant gas can, of course, be simply pumped into the chamber upon ceasing of the heating step.

Accordingly, in a second aspect the present invention consists in a method of cycling a reaction mixture between predetermined temperatures, the method involving the use of the device of the first aspect of the present invention in which samples are placed in the sample loading zones, the drive means is actuated
5 thereby spinning the rotor causing the samples to travel to the reaction zones and mix, and cyclically actuating the heating means, ceasing heating and introducing a coolant gas into the chamber via the openings.

BRIEF DESCRIPTION OF THE DRAWINGS

In order that the nature of the present invention may be more clearly understood, preferred forms of the invention will now be described with reference to the accompanying drawings in which:

5 Figure 1 and 2 show a top plan view of the rotor; and

 Figure 3 shows a schematic view of the device.

DETAILED DESCRIPTION OF THE INVENTION

As shown in Fig 1 the rotor consists of a high temperature plastic with a number of slots (1) and loading wells (2a, 2b) positioned radially. At the end of each slot there is provide a glass capillary tube (3) with the outer end sealed and
10 the inner end open to the slot. Reagents are pipetted into the loading wells, (2a, 2b) which are positioned around the inner radius of the rotor. Reagent mixture and DNA sample are separated during loading and are only mixed together when the rotor is spun, thus avoiding any hybridization before
15 thermal cycling commences.

As is perhaps better shown in Fig 2 the rotor consists of a high temperature plastic disc, which consists of upper and lower sections welded together. The lower section contains a formed slot (1) with two or more loading wells formed at the inner radius (2a, 2b). The dimensions of the rotor disc are
20 preferably identical to that of an audio Compact Disk. At the circumference of the disk the lower section also has a reaction well (3) where the samples are monitored during the run.

The upper section of the rotor has two or more holes over the loading wells, (2a, 2b) to allow reaction mixture and DNA sample to be loaded, and
25 sealed over the reaction well to form a closed vessel.

In operation reaction mixture is loaded into the loading wells (2a) and DNA sample is loaded into (2b), a cover is the clamped over the loaded wells (2a, 2b) and the rotor is the slowly rotated and brought to denaturation temperature. The rotor is then accelerated to a higher speed, sample and reagents move rapidly
30 by centrifugal force into the reaction vessel at the outer diameter of the disc.

The disc is then temperature cycled to initiate DNA amplification. While spinning the reaction products are monitored by a real time fluorescence optical system and the resulting data transferred to a computer analysis.

5 As shown in Fig 3 the device consists of an insulated base plate (1) with an insulated lid (2) containing the rotor (3) described above. The rotor (3) is driven by motor (4) which is controlled by computer (5). The position sensor (6) signals the computer when each reaction vessel is over the fluorescence detection system (7). The data acquired by the fluorescence detector is transferred to the computer for analysis.

10 To heat and cool the reaction vessels a heater (8) is placed in a duct which is fed by a fan (9), the air directed over the spinning rotor and exits via a duct (10). The rotor temperature is detected by a sensor (11) and controlled by the computer system (5).

15 It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

CLAIMS

We Claim:

1. A method of amplifying an amount of DNA present in a sample, using Polymerase Chain Reaction, Ligase Chain Reaction or any other current amplification technology, comprising the following steps:

- 5 i) adding oligonucleotide primers, deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP), and at least one of a thermostable DNA polymerase, thermostable DNA ligase, enzymatically active fragments thereof, an enzymatically active derivative thereof and a reverse
10 transcriptase, to form a Reaction mixture;
 - ii) loading the Reaction mixture into a loading well on a disposable rotor;
 - iii) loading the DNA sample into a loading well on a disposable rotor;
 - iv) placing the rotor into the centrifugal thermal cycling device and
15 spinning so as the Reaction mixture and sample DNA are moved by centrifugal force to the reaction well at the periphery of the rotor;
 - v) the mixture then undergoes thermal cycling, involving;
 - 20 (a) denaturation of the DNA into its component strands;
 - (b) annealing of the oligonucleotide primers to complementary sequences in the DNA; and
 - (c) synthesis of new DNA strands;
 - vi) Repeat step v) until a desired level of amplification has been
 achieved.
2. A method as claimed in claim 1 in which during the repetition of step v) the reaction wells are monitored by a detection means.
- 25 3. A method as claimed in claim 1 in which the Reaction mixture and Sample DNA are loaded such that Reaction mixture and DNA sample are physically separated in the loading well to prevent any hybridization occurring prior to thermal cycling.

4. A method as claimed in claim 1 where the rotor is initially spun at low speed to preheat the reaction wells, the rotor is then spun at high speed so as the Reaction mixture and sample DNA are moved by centrifugal force to the reaction well and therefore mixed together.
5. A method as claimed in claim 1 where the rotor is spun at high speed to ensure that maximum temperature uniformity is achieved between all samples at the periphery of the rotor.

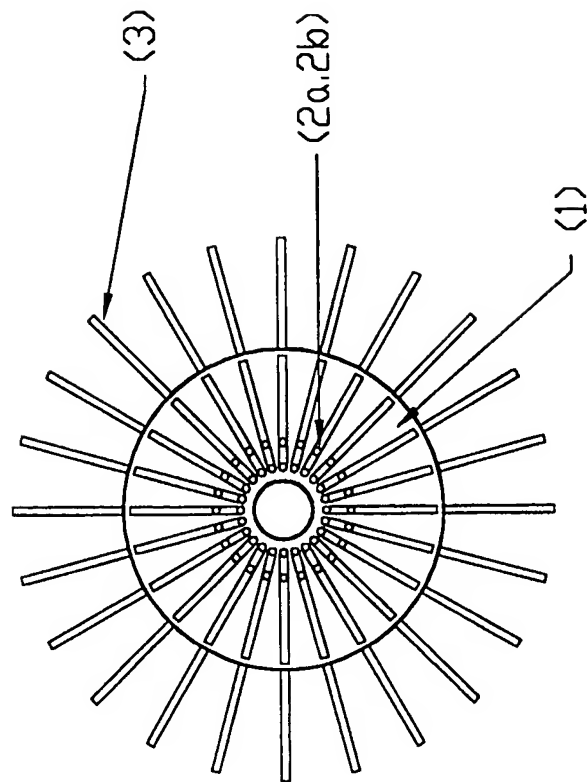


Figure 1

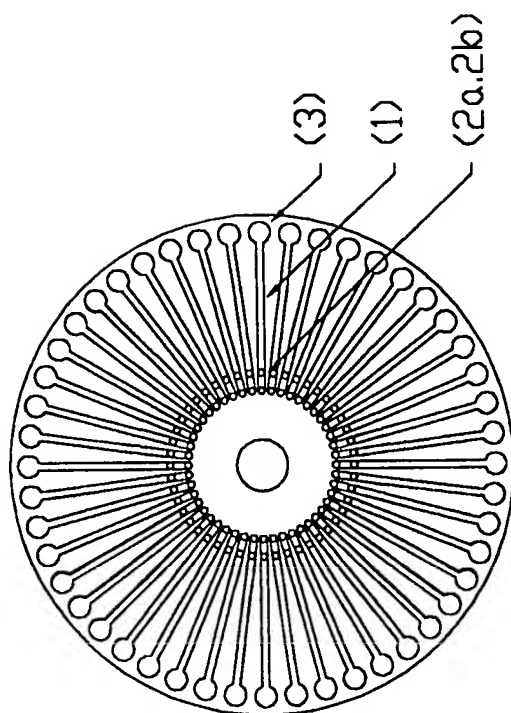


Figure 2

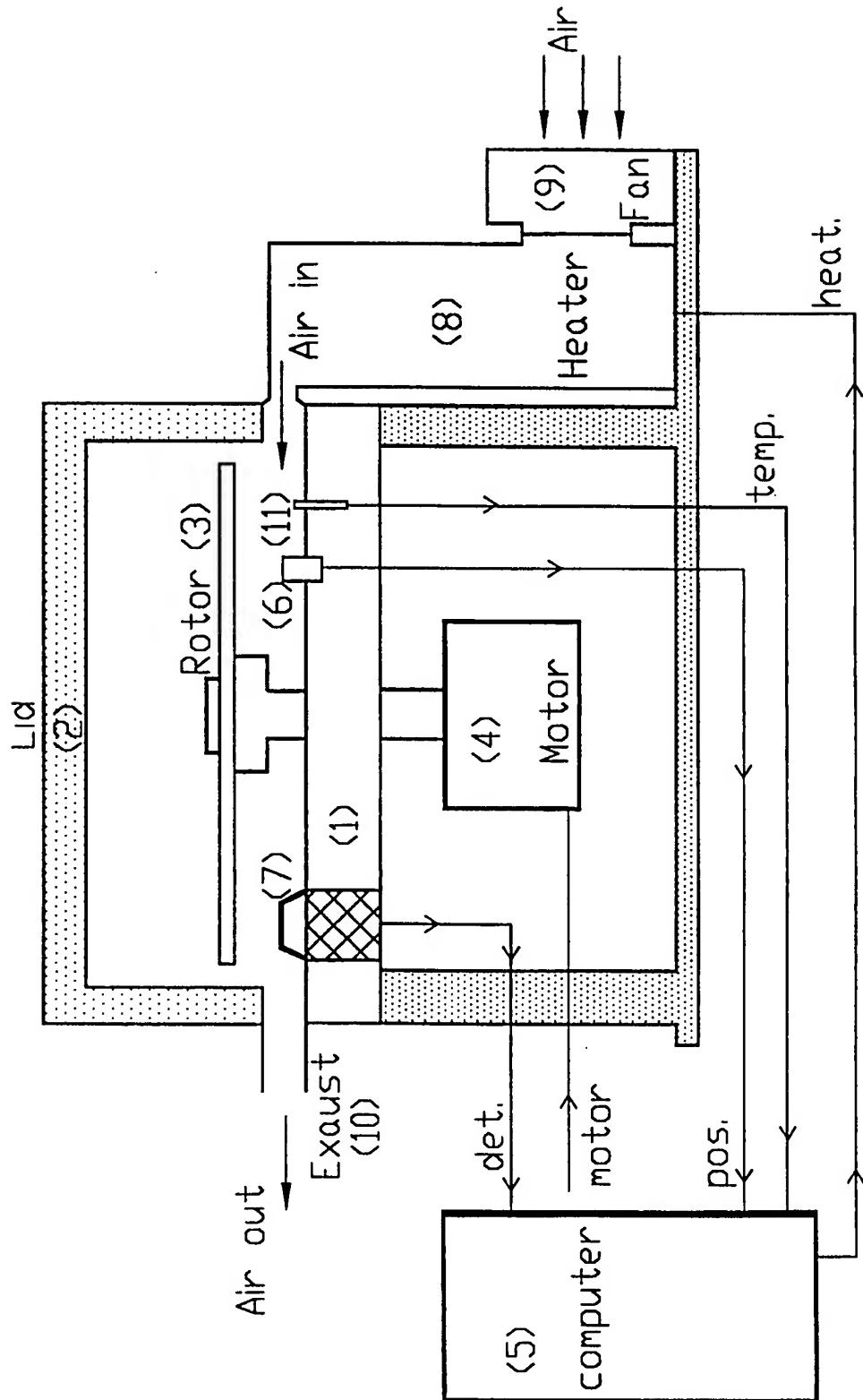


Figure 3

| A. CLASSIFICATION OF SUBJECT MATTER | | | | | | | | | | | | | | | | | | | | | | |
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| Int Cl ⁶ : C12Q 1/68; C12M 1/02, 1/38; B01L 7/00; B04B 7/00, 11/00, 15/00 | | | | | | | | | | | | | | | | | | | | | | |
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| Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPAT : + keywords CHEMICAL ABSTRACTS : + keywords | | | | | | | | | | | | | | | | | | | | | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | | | | | | | | | | | | | | | | | | | | | |
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. | | | | | | | | | | | | | | | | | | | | |
| X | EP 693560 A (BECTON DICKINSON CO) 24 January 1996 column 1 lines 24-39, column 3 lines 12-24, column 8 lines 21-48, column 16 lines 39-42, column 16 line 49 to column 19 line 27, figs 3, 15 to 18B | 1-5 | | | | | | | | | | | | | | | | | | | | |
| X | EP 169306 A (ALLIED CORP) 29 January 1986 Abstract, figs 1-3 | 1-5 | | | | | | | | | | | | | | | | | | | | |
| X | US 3856470 A (BAXTER LAB INC) 24 December 1974 column 1 line 62 to column 2 line 46, column 4 line 10 to column 5 line 10, column 7 lines 7-41, column 9 lines 3-37 | 1-5 | | | | | | | | | | | | | | | | | | | | |
| <input type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex | | | | | | | | | | | | | | | | | | | | | | |
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| Date of the actual completion of the international search 2 July 1998 | | Date of mailing of the international search report 17 JUL 1998 | | | | | | | | | | | | | | | | | | | | |
| Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929 | | Authorized officer <i>A. Achilleos</i> ANDREW ACHILLEOS Telephone No.: (02) 6283 2280 | | | | | | | | | | | | | | | | | | | | |

INTERNATIONAL SEARCH REPORT

International Application No.

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in Search Report | | | | Patent Family Member | | | |
|--|---------|----|-----------|----------------------|---------|----|---------|
| EP | 693560 | AU | 23310/95 | BR | 9503364 | CA | 2152307 |
| | | JP | 08-062225 | SG | 32408 | US | 5639428 |
| EP | 169306 | CA | 1247068 | US | 4580898 | | |
| US | 3856470 | CA | 987130 | CH | 587481 | DE | 2365286 |
| | | FR | 2213498 | GB | 1448132 | IT | 1014531 |
| | | JP | 49-104698 | NL | 7400079 | | |
| <p>END OF ANNEX</p> | | | | | | | |